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Award Number: DAMD17-00-1-0396

TITLE: Novel Synthetic Antiestrogens That Block Nuclear Estrogen Receptor Function Through Plasma Membrane Localization

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REPORT DATE: May 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2003	Annual Summary (1 May 00 - 30 Apr 03)

4. TITLE AND SUBTITLE
Novel Synthetic Antiestrogens That Block Nuclear Estrogen Receptor Function Through Plasma Membrane Localization

5. FUNDING NUMBERS
DAMD17-00-1-0396

6. AUTHOR(S):
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Hormonally responsive breast cancers that respond favorably to antiestrogens such as tamoxifen often become resistant to this treatment. We are working to identify novel antiestrogens that promote plasma membrane localization of estrogen receptors (ERs). Thus far, we have not successfully recruited ERs to plasma membranes. To probe the failure of the ER recruitment system we studied the ability of membrane-anchored ligands to recruit other intracellulary-expressed proteins that might provide a simpler model system of the ER. Studies of a cholesterylamine-biotin chimera led to the discovery that this compound promotes streptavidin (SA, expressed in Jurkat lymphocytes, fused to Green Fluorescent Protein (GFP)) and Apo-1) recruitment to the plasma membrane. This biotin - SA system provides a great model for further studies of hER membrane recruitment. Because such compounds "dimerize" SA with the plasma membrane, we were interested in compounds that could dimerize SA with the estrogen receptor. Toward that end, we developed a novel chemical inducer of dimerization comprising β-estradiol linked to biotin. This compound potently dimerizes streptavidin and the estrogen receptor in the nucleus of yeast. This result sheds more light on methods for manipulating the estrogen receptor in living cells.

14. SUBJECT TERMS:
drug discovery, steroid hormones, estrogen antagonists, estrogen receptor, organic synthesis, chemotherapy, lipidation

15. NUMBER OF PAGES
16

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

Hormonally dependant breast cancers that respond favorably to antiestrogens such as tamoxifen often become resistant to this treatment. Novel antiestrogens that inhibit estrogen receptor-mediated gene expression through novel mechanisms of action may provide more effective therapeutics. We are developing novel anti-estrogens that function by targeting estrogen receptors (ERs) to the plasma membrane. Thus far, synthetic compounds comprising β-estradiol conjugated to cholesterol and cholesterylamine have been unsuccessful in recruiting ERs to membranes (Appendix A).[1] To address this failure we have synthesized a novel compound (**1**) that replaces β-estradiol with biotin and has the potential to target streptavidin (SA) to membranes. Treatment of Jurkat lymphocytes expressing SA fused to green fluorescent protein (GFP) and Apo-1 with streptaphage (**1**, 10 μM) revealed recruitment of the cytosolic protein to the plasma membrane in most of the observed cells (Figure 1). This fusion protein was designed to initiate apoptosis upon membrane recruitment. These results of these studies are encouraging for the continued development of compounds capable of targeting ERs to membranes.

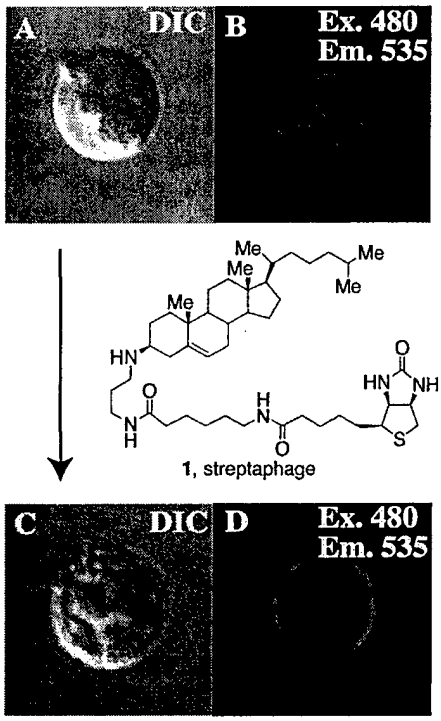


Figure 1. Epifluorescence micrographs of cells expressing SA-Apo-1-GFP. Panels A, B: No streptaphage (**1**) added. Panels C, D: Streptaphage (**1**, 10 μM) added for 10 h.

Body

Streptaphage was, in effect, designed to dimerize a cytosolic protein with the plasma membrane. While this design has many potential applications in biology, compounds capable of dimerizing two proteins in the nucleus of living cells provide important tools for probing many other biological processes.[2] Chemical inducers of protein dimerization (CIDs) have been used to control intracellular signal transduction

pathways, protein subcellular localization, and gene expression.[3-6] This approach has been used to identify protein targets of small molecule natural products.[7] Pioneering work by Liu linked the steroid dexamethasone to the natural product FK506 to identify the protein target FKBP by screening this chimeric compound against a genetically encoded library of proteins in a yeast three hybrid system.[7] This system employed an engineered glucocorticoid receptor (GR) protein as a DNA-bound platform that displayed dexamethasone-tethered FK506 to target proteins that activate gene expression upon binding.

Although screening natural products against protein targets with yeast three hybrid systems is a potentially elegant alternative to traditional affinity chromatography methods, dexamethasone derivatives are limited in this regard by the relatively low activity of glucocorticoids in recombinant yeast.[8] This low activity relates in part to the observation that yeast-expressed GR proteins bind dexamethasone with >10^3-fold lower affinity than GR proteins expressed in mammalian cells.[9]

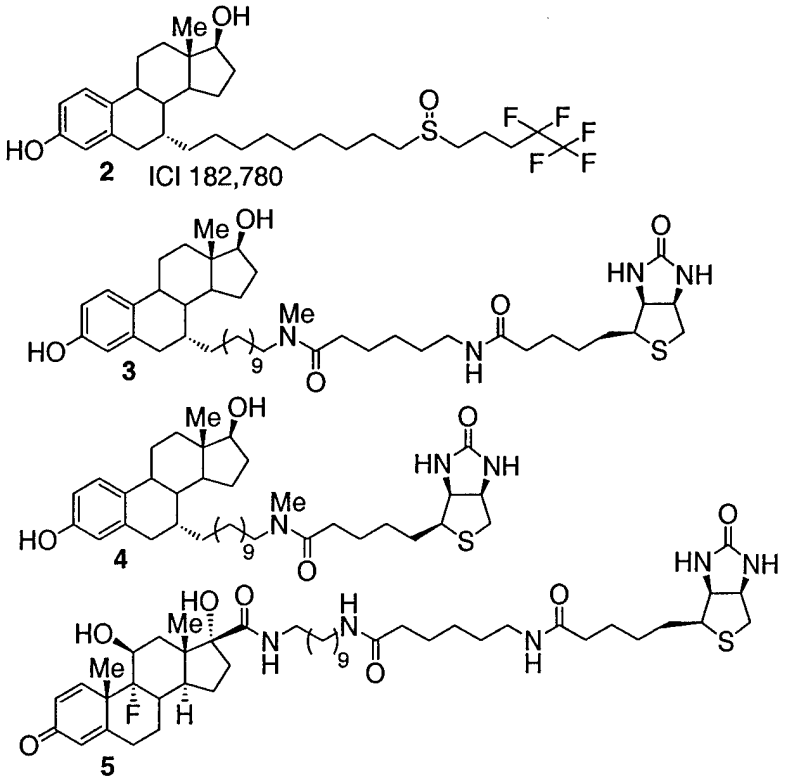
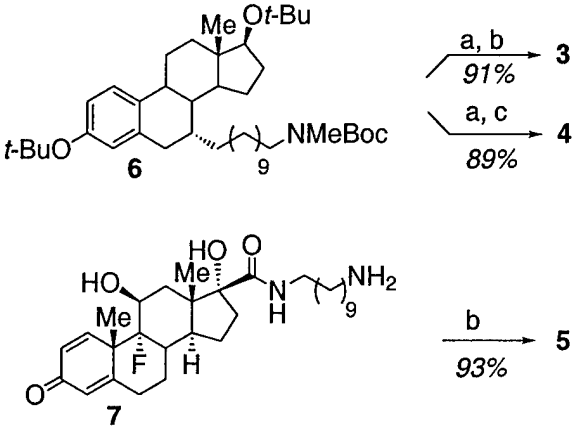


Figure 2. Structure of 7α-substituted estradiol derivatives.

In contrast to glucocorticoid-based yeast three-hybrid systems, steroidal estrogens are highly active in yeast systems,[1] and 7α-substituted estradiol derivatives such as the antiestrogen ICI 182,780 (**2**, Figure 2) bind tightly to both the estrogen receptor α (ERα, K_d~1.0 nM) and estrogen receptor β (ERβ, K_d~3.6 nM) isoforms.[10] Furthermore, high resolution X-ray crystal structures of these proteins bound to cognate ligands are available for design of CIDs.[11] To investigate these potential advantages for the analysis

of natural products in yeast three-hybrid-based systems, we employed the previously reported protected 7α-substituted β-estradiol derivative **6**[1] to synthesize the chimeric 7α-substituted β-estradiol derivatives **3** and **4** linked to the natural product biotin (Scheme 1). The dexamethasone-biotin derivative **5** was also prepared as shown in Scheme 1 from the known compound **7**[7] to directly compare yeast three-hybrid systems based on GR-dexamethasone and ER-estradiol molecular recognition in vivo.

Scheme 1. Synthesis of 7-α-substituted estradiol chimeras **3**, **4**, and **5**.



To analyze ligand-mediated protein heterodimerization in vivo, yeast were engineered to express ERβ LBD and SA fusion proteins as shown in Figure 3. In this novel yeast three hybrid system, the bacterial LexA protein[12] was fused to the N-terminus of the steroid receptor to anchor this protein on DNA sites that control expression of a reporter gene, and the bacterial B42 activation domain (AD)[12] was fused to the SA C-terminus to activate gene expression if this protein was brought into proximity of the

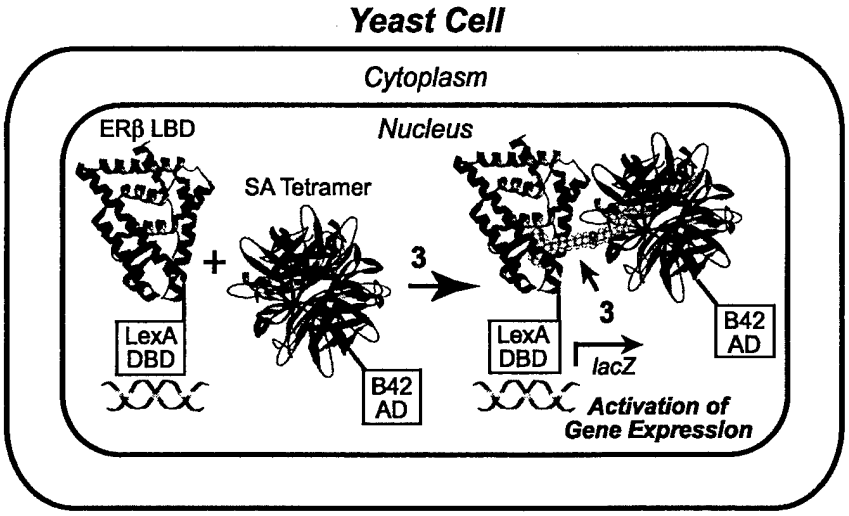


Figure 3. Schematic of the ER-SA yeast three hybrid assay showing a hypothetical model of the ternary complex. Addition of ligand **3** heterodimerizes the DNA-bound LexA-ER fusion protein and the SA-B42 fusion protein to activate expression of a *lacZ* reporter gene.

LexA fusion protein by a small molecule-protein interaction. Analogous yeast three hybrid assays were constructed by substituting the ERβ LBD with the ERα LBD and the GR LBD.

Addition of ligands **3** and **4** to yeast three hybrid systems and analysis of ligand-mediated gene expression provided the dose-response curves shown in Figure 4. Ligand **3** potently activated gene expression in yeast expressing either the ERα (~380-fold activation at 10 μM, Panel A) or ERβ LBD (~450-fold at 10 μM, Panel B) compared with levels of activation in the absence of ligand. Surprisingly, the lower affinity mutant SA Y43A and SA W120A proteins were nearly as effective as SA W.T. in mediating this dose-response (Figure 4), revealing that moderate-affinity interactions can be detected with this approach. Surprisingly, the W79A mutant was inactive in this assay.

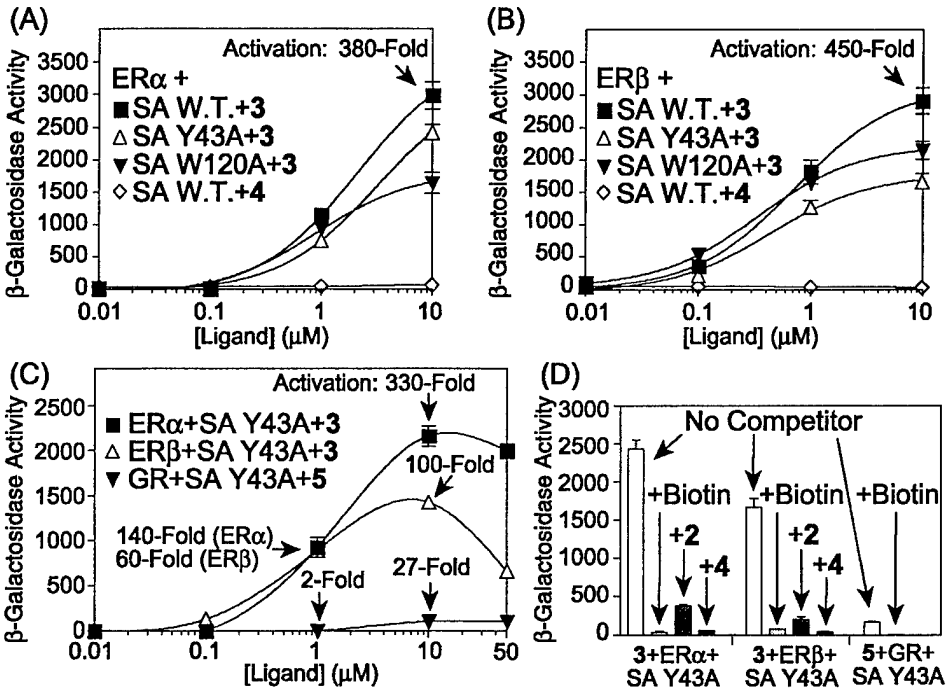


Figure 4. Dose-response curves and competition experiments. Panels A-C: Fold activation = observed β-Galactosidase activity / β-Galactosidase activity without ligand. Panel D: [Ligand]=10 μM. [Biotin] =100 μM. [**2**] and [**4**]=50 μM.

Activation of gene expression by dexamethasone derivative **5** was directly compared with **3** using SA Y43A expressed in three hybrid systems due to the predicted high affinity of this SA protein for biotin derivatives and favorable cellular growth characteristics. Remarkably, ligand **3** was much more potent (ERβ-SA_{Y43A} EC_{50}=700 nM;

9-fold activation at 100 nM; 60-fold {140-fold for ERα} activation at 1 μM) than activation by **5** (GR-SA_{Y43A} EC_{50}=3.6 μM; 2-fold activation at 1 μM, 27-fold activation at 10 μM). Moreover, the absolute magnitude of response with **3** was up to 70-fold greater than **5** at 1 μM (Figure 4, Panel C). At the high concentration of 50 μM, ligand **3** was sufficiently potent to partially competitively inhibit reporter gene expression. Although not commonly observed in three hybrid systems, this auto-inhibition is predicted to occur if all of the protein binding sites become occupied by excess ligand. Competition experiments with free biotin and the inactive **4** confirmed the specificity of these interactions (Figure 4, Panel D).

These results indicate that 7α-substituted derivatives of β-estradiol are highly potent and effective activators of gene expression in living yeast cells (Appendix B. Coupling these compounds to natural products may facilitate the identification of protein targets from cDNA libraries expressed in yeast three hybrid systems.

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Key Research Accomplishments:

• Initial testing of streptaphage revealed that it is capable of recruiting a streptavidin – green fluorescent protein fusion (expressed in Jurkats) to the plasma membrane in a moderate percentage of analyzed cells (40 -50 %).

• Further testing of streptaphage revealed that it is capable of recruiting a streptavidin – Apo-1-green fluorescent protein fusion (expressed in Jurkats) to the plasma membrane in a higher percentage of analyzed cells (80 - 90 %).

• Synthesized novel 7-α-substituted β-estradiol - biotin chimeras.

• Synthesized a novel dexamethasone - biotin chimera.

• Analyzed the longer linker estradiol - biotin chimera in a yeast three-hybrid assay and found that it is a potent dimerizer of the estrogen receptor and streptavidin proteins.

Reportable Outcomes:

Harry and Catherine Dalalian Graduate Fellowship in Organic Chemistry, 2003

1. Hussey, S. L., He, E., Peterson, B. R. "A Synthetic Membrane-Anchored Antigen Efficiently Promotes Uptake of Antifluorescein Antibodies and Associated Protein A by Mammalian Cells" *J. Am. Chem. Soc.* **2001**, 123, 12712-12713.

2. Hussey, S. L., He, E., Peterson, B. R. "Synthesis of Chimeric 7α-Substituted Estradiol Derivatives Linked to Cholesterol and Cholesterylamine" *Org. Lett.* **2001**, 4 (3), 415-418.

3. Hussey, S. L., Peterson, B. R. "Efficient Delivery of Streptavidin Conjugates to Mammalian Cells: Clathrin-Mediated Endocytosis Regulated by a Synthetic Ligand" *J. Am. Chem. Soc.* **2002**, *124,* 6265-6273.

4. Hussey, S. L.; Muddana, S. S.; Peterson, B. R. "Synthesis of a β-Estradiol-Biotin Chimera that Potently Heterodimerizes Estrogen Receptor and Streptavidin Proteins in a Yeast Three Hybrid System." *J. Am. Chem. Soc.* **2003**; ASAP Article.

Conclusions:

It is known that breast cancers that respond favorably to antiestrogens such as tamoxifen will develop resistant to tamoxifen chemotherapy. Antiestrogens that function through novel mechanisms of action may be able to halt the growth of tamoxifen-resistant tumors. This research is oriented towards the identification of novel antiestrogens that promote plasma membrane localization of estrogen receptors. The successful development of this approach may fully block ER function and yield agents that kill tamoxifen-refractory breast cancers. Thus far, membrane-recruitment of ERs has been difficult to achieve. Membrane-localization studies involving a simpler model system were continued with a new biotin – cholesterylamine conjugate. This compound recruited SA fusion proteins to the plasma membrane in a good percentage of the mammalian cells analyzed.

Furthermore, the activity of these compounds leads to the discovery and synthesis of a novel β-estradiol - biotin conjugate that potentially dimerizes the estrogen receptor and streptavidin proteins in yeast. This system can be used to discover novel β-estradiol binding proteins that might provide novel targets for breast cancer therapeutics.

There has been slight deviation from the original statement of work in the final year of the described research. However, the exciting development of the SA-recruitment system and subsequent three-hybrid chemical inducer of dimerization has warranted such changes.

References: Please see body of report.

Appendices: Please see attached publications.

ORGANIC LETTERS

2002
Vol. 4, No. 3
415–418

Synthesis of Chimeric 7α-Substituted Estradiol Derivatives Linked to Cholesterol and Cholesterylamine

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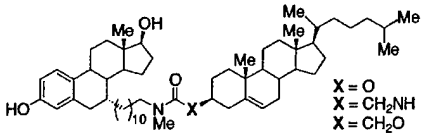
Received November 26, 2001

ABSTRACT



We report the synthesis of 7α-substituted β-estradiol derivatives bearing side chains terminated with cholesterol and 3β-cholesterylamine. These chimeric compounds were designed to exhibit high affinity for estrogen receptors (ERs) and cellular plasma membranes to potentially enable regulated uptake of ERs by mammalian cells. Evaluation with recombinant yeast reporting compound-mediated ER dimerization revealed potencies similar to the antiestrogen ICI 182780. Compounds that efficiently deliver dominant negative ERs into cells may provide novel therapeutics against breast cancers.

Compounds that enable the regulated delivery of small molecules, proteins, and DNA to mammalian cells are critical to the effectiveness of therapeutics and molecular probes.[1] Since macromolecules do not efficiently penetrate cell membranes, the delivery of these compounds to cells is typically mediated by lipids[2–5] or cationic polymers[6–9] that modulate the chemical properties of their cargo prior to addition of complexes or conjugates to cells. Alternatively, cellular membranes can be chemically altered to facilitate macromolecular uptake.[10–12] As an example of this latter approach, we recently reported[13] the synthesis of the fluorescent "memtigen" (membrane-anchored antigen) **1** (Figure 1), which strongly associates with cellular plasma membranes. When added to mammalian cells, **1** efficiently promotes uptake of macromolecular antifluorescein antibodies and associated protein complexes in nearly 100% of viable cells.

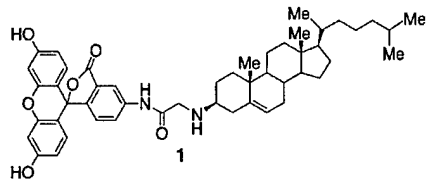


Figure 1. Structure of a previously reported fluorescein–cholesteramine chimera (**1**) that enables uptake of antifluorescein antibodies and associated protein complexes by mammalian cells.

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10.1021/ol0171261 CCC: $22.00
Published on Web 01/05/2002

We report here the synthesis of structurally related 7α-substituted derivatives of β-estradiol (**2**) linked to cholesterol and cholesterylamine. The antiestrogens ICI 182780 (**3**)[14] and ICI 164384 (**4**)[15] (Figure 2) provided models for the

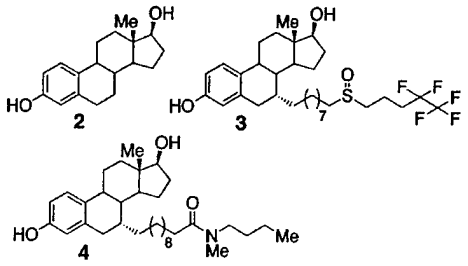


Figure 2. Structures of the estrogenic steroid hormone β-estradiol (**2**), the antiestrogen ICI 182780 (**3**), and the antiestrogen ICI 164384 (**4**).

design of compounds with high affinity for estrogen receptor (ER) proteins. Antiestrogens such as tamoxifen, raloxifene, **3**, and **4**, are employed clinically to treat hormonally responsive breast cancers dependent on estrogens such as **2** to proliferate.[16] The proliferation of these cancers is controlled by ERs α and β, which are transcription factor proteins that regulate gene expression in the cell nucleus.[16-18]

Although antiestrogens **3** and **4** comprise potent competitive antagonists of estrogen receptors,[14,15] most breast cancers eventually become resistant to these types of antihormone therapeutics.[19] As an alternative therapeutic approach, the delivery of transcriptionally altered ERs, termed dominant negative mutants, to breast cancer cells holds promise as a strategy to treat breast cancer.[20] However, current methods of delivery of estrogen receptors are either inefficient or require the use of a recombinant virus, which limits the therapeutic potential of this approach.[20,21]

As a preliminary step directed at investigating whether small molecules might be employed to deliver ERs into mammalian cells, we report here the synthesis of compounds **5**–**8** (Figure 3). Recent X-ray crystal structures of ERβ-

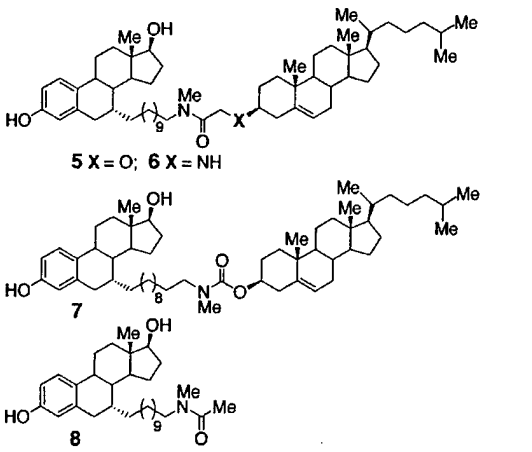
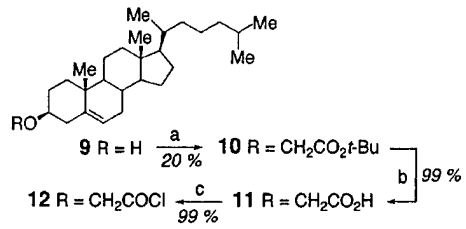


Figure 3. Structures of synthetic targets.

bound **4** revealed that whereas the steroid is buried in the protein interior, the amide nitrogen atom of the side chain is solvent-exposed,[22] suggesting that this would be an excellent point of attachment for cholesterol derivatives. Cholesterol (**9**) derivatives were chosen for investigation because cholesterol is an abundant plasma-membrane-associated steroid that controls membrane fluidity[23] and is covalently linked to proteins involved in cellular signaling[24] and synthetic derivatives can enable protein uptake by mammalian cells.[13]

The synthesis of compounds **5**–**7** required cholesterol-derived electrophiles **12**–**14**. The acid chloride **12** was prepared from cholesterol (**9**) in three steps as shown in Scheme 1. As shown in Figure 4, cholesteryl chloroformate

Scheme 1[a]



[a] Reagents and conditions: (a) NaH, *tert*-butylbromoacetate, THF, reflux. (b) HCO_2H, Et_2O, 65 °C. (c) $SOCl_2$, CH_2Cl_2, reflux.

(**13**) was commercially available, and the protected cholesterylamine (**14**) was prepared as previously described.[13]

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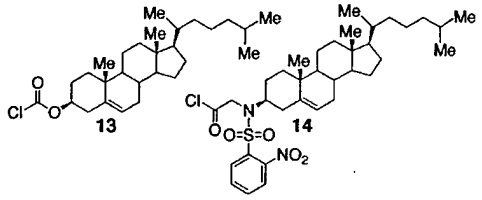
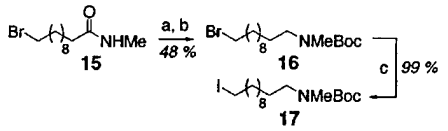


Figure 4. Structures of commercially available cholesteryl chloroformate (**13**) and the previously described acid chloride (**14**).

Steroid side chain precursors were prepared from the known[25] *N*-methylamide **15** as shown in Scheme 2. Reduc-

Scheme 2[a]

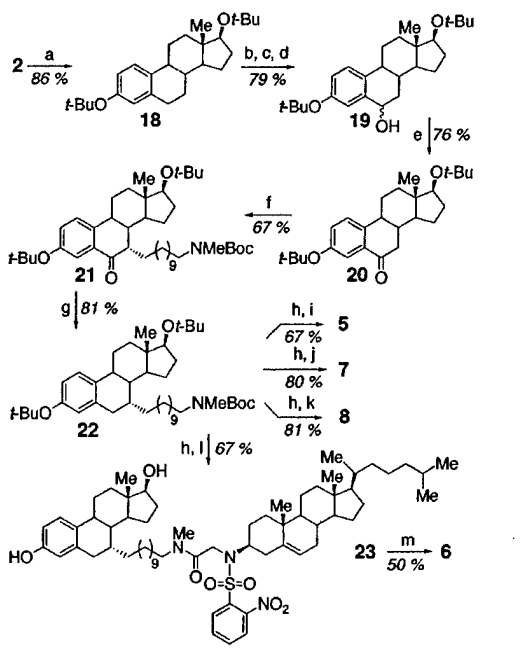


[a] Reagents and conditions: (a) DIBAL, CH_2Cl_2, 25 °C. (b) $(Boc)_2O$, DIEA, CH_2Cl_2, 25 °C. (c) NaI, acetone, reflux.

tion of the amide, protection of the resulting amine, and conversion to the iodide under Finkelstein conditions afforded the *tert*-butylcarbamate-protected *N*-methylamine **17** (Scheme 2).

In contrast to other syntheses of 7α-substituted estradiol derivatives,[26,27] which have primarily employed tetrahydropyranyl (THP) ethers for alcohol protection,[28–34] we installed *tert*-butyl ether protecting groups for the preparation of this class of compounds. This approach avoided generation of mixtures of diastereomers that limit facile assignment of side chain stereochemistry by NMR. Protection of **2** as the di-*tert*-butyl ether[35] was accomplished in good yield using a heterogeneous mixture of isobutylene and acidic Amberlyst resin in a sealed flask (Scheme 3).

Scheme 3[a]



[a] Reagents and conditions: (a) Isobutylene, acidic Amberlyst-15, CH_2Cl_2, 25 °C. (b) LDA, *t*-BuOK, THF, −78 °C. (c) $B(OMe)_3$, 0 °C. (d) H_2O_2, H_2O, 25 °C. (e) NaOCl, TEMPO, KBr, H_2O, CH_2Cl_2, 25 °C. (f) *t*-BuOK, **17**, THF, 0 °C. (g) 10% Pd (C), H_2, ethanol, 65 °C. (h) Trichloroacetic acid, CH_2Cl_2, 25 °C. (i) **12**, DIEA, THF, 25 °C. (j) **13**, DIEA, THF, 25 °C. (k) Ac_2O, DIEA, THF, 25 °C. (l) **14**, DIEA, THF, 25 °C. (m) PhSH, K_2CO_3, CH_3CN, 25 °C.

Compound **18** was deprotonated under well precedented[28,36,37] "superbase" conditions employing a 1:1 ratio of potassium *tert*-butoxide and lithium diisopropylamide. This anion was trapped with trimethyl borate to yield an intermediate borate ester that was oxidized with hydrogen peroxide to epimeric alcohols **19**. These epimers were further oxidized to ketone **20** with aqueous sodium hypochlorite, including TEMPO free radical and KBr as catalysts.[38]

Deprotonation of **20** with potassium *tert*-butoxide generated the corresponding enolate, which was alkylated with iodoalkane **17** to furnish ketone **21** as a single epimer in good yield. A minor O-alkylation product could also be isolated. Two-dimensional NMR experiments unambiguously confirmed the configuration of the 7α-side chain of **21** by detection of a nuclear Overhauser effect (NOE) between protons H_7 and H_8 and the absence of a NOE between H_7 and H_9 (Figure 5).

Hydrogenolysis of **21** over palladium on carbon afforded **22** in high yield. Complete removal of the acid-labile

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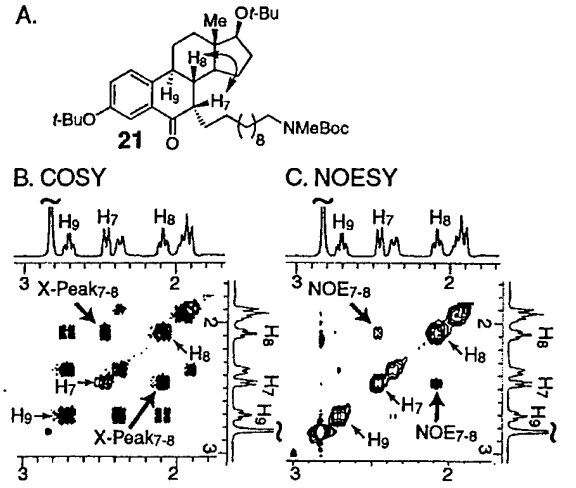

Figure 5. Assignment of 7α-stereochemistry to compound **21** by 2-D NMR. (A) Structure of **21** depicting the diagnostic NOE between H_7 and H_8. (B) COSY spectrum of B-ring proton resonances. (C) NOESY spectrum of this region.

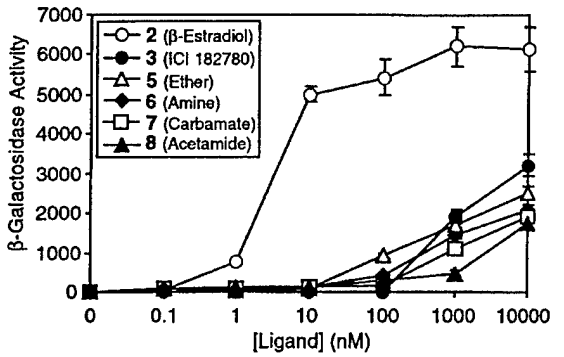

Figure 6. Dose-dependent activation of gene expression in whole yeast cells engineered to report compound-induced dimerization of ERα.

protecting groups with 10% trichloroacetic acid was followed by acylation of the side chain amine with **12**, **13**, acetic anhydride, and **14** to yield **5**, **7**, **8**, and **23** as shown in Scheme 3. The 2-nitrobenzene sulfonamide protecting group on **23** was removed with deprotonated thiophenol to provide **6**.

The ability of these 7α-substituted β-estradiol derivatives to interact with ERα was assessed by comparison with β-estradiol (**2**) and the antiestrogen ICI 182780 (**3**) in a yeast whole cell assay[39,40] that reports both estrogen- and antiestrogen-induced dimerization of ERα by activating expression of the enzyme β-galactosidase. Although competitive binding assays with purified ERα and radiolabeled or fluorescent estrogen probes might have provided a more quantitative comparison, recombinant yeast provide well-precedented assays[39,40] for analysis of compound-mediated ER dimerization. This yeast-based assay provided a simple, rapid, inexpensive, and nonradioactive method for generation of initial biological activity data.

As shown in Figure 6, these experiments revealed that compounds **5**–**8** exhibit activities nearly identical to that of ICI 182780 (**3**). Remarkably, the cholesterol derivatives **5**–**7** did not differ from the acetylated control compound **8** in ability to induce ERα dimerization, indicating that these compounds exhibit substantial affinity for estrogen receptors. Future studies will investigate the ability of lipidic estrogens to promote uptake of estrogen receptors by mammalian cells.

Acknowledgment. We thank the National Institutes of Health (CA83831) for financial support. S.L.H. thanks the Department of Defense for a predoctoral fellowship. We thank Dr. B. Katzenellenbogen for the gene encoding ERα.

Supporting Information Available: Experimental procedures and characterization data for new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Synthesis of a β-Estradiol-Biotin Chimera that Potently Heterodimerizes Estrogen Receptor and Streptavidin Proteins in a Yeast Three-Hybrid System

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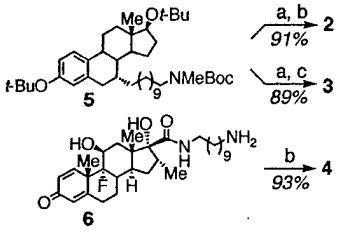
Small molecules that dimerize proteins in living cells provide important tools for probing diverse biological processes.[1] Chemical inducers of protein dimerization (CIDs) have been used to control intracellular signal transduction pathways, protein subcellular localization, and gene expression.[2] This approach has also been used to identify protein targets of small molecule natural products.[3] Pioneering work by Liu linked the steroid dexamethasone to the natural product FK506 to identify the protein target FKBP by screening this chimeric compound against a genetically encoded library of proteins in a yeast three-hybrid system.[3] This system employed an engineered glucocorticoid receptor (GR) protein as a DNA-bound platform to display dexamethasone-tethered FK506 to target proteins that activate gene expression upon binding.

Although screening natural products against protein targets with yeast three-hybrid systems is a potentially elegant alternative to traditional affinity chromatography methods, dexamethasone derivatives are limited in this regard by the relatively low activity of glucocorticoids in recombinant yeast.[4] This low activity relates in part to the observation that yeast-expressed GR proteins bind dexamethasone with $>10^3$-fold lower affinity than GR proteins expressed in mammalian cells.[5] In contrast, steroidal estrogens are highly active in yeast systems,[6] and 7-α-substituted estradiol derivatives such as the antiestrogen ICI 182,780 (**1**) bind tightly to both the estrogen receptor α (ER-α, $K_d \approx 1.0$ nM) and the estrogen receptor β (ER-β, $K_d \approx 3.6$ nM) isoforms.[7] Furthermore, high-resolution X-ray crystal structures of these proteins bound to cognate ligands are available for design of CIDs.[8] To investigate these potential advantages for the analysis of natural products in yeast-based systems, we employed the previously reported protected 7-α-substituted β-estradiol derivative **5**[6b] to synthesize the chimeric 7-α-substituted β-estradiol derivatives **2** and **3** linked to the natural product biotin (Scheme 1). Other biotinylated β-estradiol derivatives have also been reported in the literature.[9] Biotin was chosen because molecular recognition by the bacterial streptavidin (SA) protein has been extensively characterized.[10] Moreover, interactions between biotin and streptavidin have not been previously investigated in a yeast three-hybrid system, and biotin provides a simple model of more complex natural products. The dexamethasone-biotin derivative **4** was prepared from **6**[3] (Scheme 1) to directly compare yeast three-hybrid systems on the basis of GR-dexamethasone and ER-estradiol molecular recognition in vivo.

The availability of X-ray crystal structures of the ligand binding domain (LBD) of ER-β (PDB code 1HJ1)[8] bound to the antiestrogen ICI 164,384 (structurally similar to **1**) and tetrameric streptavidin (PDB code 1SWR)[11] bound to biotin enabled construction of a simple molecular model of a ternary protein−ligand complex (Figure 1, see Supporting Information for details). Actual protein−ligand interactions formed in vivo will be much more complex; estrogen binding to ER monomers promotes ER homodimerization, and tetrameric SA binds four biotin ligands. Modeling suggested that the longer linker of ligand **2** as compared with ligand **3** would be necessary to effectively bridge both binding sites and heterodimerize these proteins.

Scheme 1 [a]



[a] (a) HCl (aq.)/dioxane (1:9). (b) D-Biotinamidocaproate NHS ester, DIEA, CH_2Cl_2/MeOH or THF. (c) D-Biotin NHS ester, DIEA, CH_2Cl_2/MeOH.

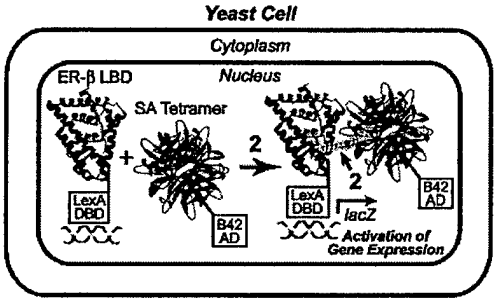
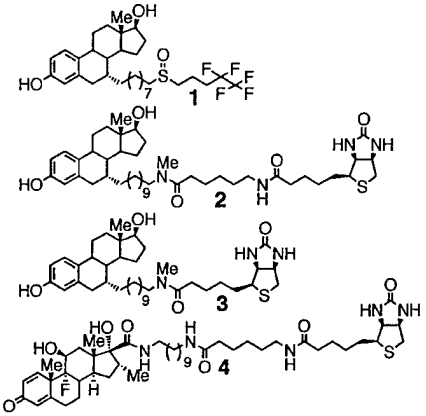


Figure 1. Schematic of the ER-SA yeast three-hybrid assay showing a hypothetical model of the ternary complex. Addition of ligand **2** heterodimerizes the DNA-bound LexA-ER fusion protein and the SA-B42 fusion protein to activate expression of a *lacZ* reporter gene.



To analyze ligand-mediated protein heterodimerization in vivo, yeast were engineered to express ER-β LBD and SA fusion proteins as shown in Figure 1. In this novel yeast three-hybrid system, the DNA binding domain (DBD) of the bacterial LexA protein[12] was fused to the N-terminus of the steroid receptor to anchor this protein

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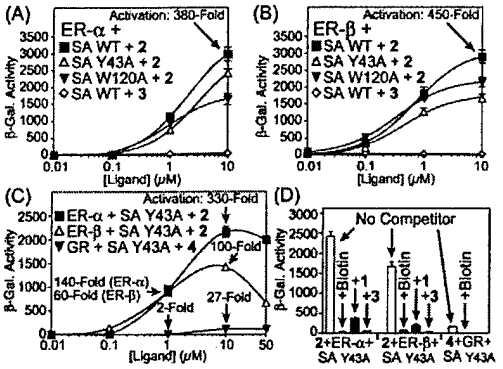

Figure 2. Dose–response curves and competition experiments. Panels A–C: Fold activation = observed β-Gal. activity/β-Gal. activity without ligand. Panel D: [Ligand] = 10 μM. [Biotin] = 100 μM. [**1**] and [**3**] = 50 μM.

on DNA sites that control expression of a *lacZ* (β-galactosidase) reporter gene. The bacterial B42 activation domain (AD)[12] was fused to the SA C-terminus to activate gene expression upon small molecule-mediated heterodimerization with the ER-β-LexA fusion protein. Analogous three-hybrid assays were constructed by substituting the ER-β LBD with the ER-α LBD and the GR LBD.

Functional SA has been expressed in bacteria fused to an N-terminal T7 peptide tag to facilitate protein folding.[13] We employed this approach to express functional SA proteins in yeast. However, expression of wild-type (WT) SA fused to the B42 AD resulted in a substantial reduction in the rate of yeast cell growth (data shown in the Supporting Information), presumably due to the high affinity ($K_d \approx 100$ fM)[11] of SA for endogenous biotin, which is an essential vitamin. In an attempt to attenuate this toxicity, site directed mutagenesis was employed to generate the known lower-affinity SA mutants: SA Y43A ($K_d \approx 100$ pM)[10a] and SA W120A ($K_d \approx 100$ nM).[11] As expected, yeast expressing these mutant proteins exhibited substantially enhanced rates of cellular growth (data shown in the Supporting Information).

Addition of ligands **2** and **3** to yeast three-hybrid systems and analysis of ligand-mediated gene expression provided the dose–response curves shown in Figure 2 (panels A and B). Ligand **2** potently activated gene expression in yeast expressing either the ER-α (~380-fold activation at 10 μM, panel A) or the ER-β LBD (~450-fold at 10 μM, panel B) as compared with levels of gene expression in the absence of ligand. Surprisingly, the lower affinity mutant SA Y43A and SA W120A proteins were nearly as effective as SA WT in mediating this dose–response (Figure 2), revealing that moderate-affinity interactions can be detected with this approach. Analysis of the toxic SA WT protein in this three-hybrid system was possible because expression of this protein was controlled by the galactose-inducible Gal1 promoter.[12] As predicted from molecular modeling, ligand **3** did not significantly activate gene expression in ER-SA three-hybrid assays.

Yeast three-hybrid systems expressing cognate steroid receptor proteins and SA Y43A were employed to directly compare activation of gene expression by dexamethasone derivative **4** and β-estradiol derivative **2**. Remarkably, ligand **2** was more potent and much more active (ER-β-SA_{Y43A} EC_{50} = 700 nM; 9-fold activation at 100 nM; 60-fold (ER-β) to 140-fold (ER-α) activation at 1 μM) than **4** (GR-SA_{Y43A} EC_{50} = 3.6 μM; 2-fold activation at 1 μM, 27-fold activation at 10 μM). Moreover, the absolute magnitude of the response with **2** was up to 70-fold greater than that with **4** at 1 μM (Figure 2, panel C). At the high concentration of 50 μM, ligand **2** was sufficiently potent to partially competitively inhibit reporter gene expression. Although not commonly observed in three-hybrid systems, this autoinhibition is predicted to occur if all protein binding sites become occupied by excess ligand. Competition experiments confirmed that biotin, **1**, and **3** are antagonists, establishing the specificity of these interactions (Figure 2, panel D).

Estrogen receptors expressed in yeast homodimerize upon addition of 7-α-substituted estradiol derivatives.[6b,14] Thus, substitution of SA-B42 with B42-ER in the three-hybrid assay enables evaluation of the cell permeability of compounds linked to β-estradiol. This analysis of compounds **1**–**3** revealed similar levels of ligand-mediated ER dimerization (data provided in the Supporting Information). This approach provides information regarding compound cellular permeability prior to screening of compounds against libraries of proteins.

These results indicate that 7-α-substituted derivatives of β-estradiol can be employed as highly effective activators of gene expression in living yeast cells. Coupling these compounds to biologically active small molecules may facilitate the identification of cognate protein targets expressed in yeast three-hybrid systems.

Acknowledgment. We thank Drs. B. Katzenellenbogen, S. Nilsson, J. Liu, and T. Sano for the receptor genes. We thank the National Institutes of Health (R01-CA83831) and American Cancer Society (RSG-02-025-01) for financial support. S.L.H. thanks the Department of Defense for a predoctoral fellowship.

Supporting Information Available: Experimental procedures, additional data and control experiments, and characterization data for new compounds (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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JA0293305